Cytogenetic Analysis in Prenatal Diagnosis

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Chromosome analysis is the single most frequent test used in laboratory prenatal diagnostic studies. I summarize the current status of the field, including diagnostic problems in the laboratory and the clinical problems associated with communicating unexpected laboratory findings. I explore the effect of molecular genetics on these issues and its possible future effects on the entire practice of prenatal diagnosis as it relates to the risk for chromosome nondisjunction (trisomy). I also discuss the use of cytogenetic analysis in the prenatal diagnosis of certain inherited genetic diseases.

(Schonberg SA: Cytogenetic analysis in prenatal diagnosis, *In* Fetal Medicine [Special Issue]. West J Med 1993; 159:360-365)

Chromosome abnormality, represented primarily by numerical change (trisomy resulting from nondisjunction or triploidy due to dispermy), is the single greatest contributor to prenatal morbidity and mortality. About 30% of recognized cases of embryonic and fetal death is due to chromosome abnormality. The incidence in unrecognized pregnancies—those resulting in early fetal demise—may be much higher.[1] As discussed elsewhere in this issue,* an increased risk of nondisjunction associated with either increased maternal age or positive maternal serum screening tests represents the most frequent indication for prenatal diagnosis. Laboratory methods for cell culture of amniotic fluid and chorionic villi, or in exceptional situations, fetal blood specimens, and for preparing "chromosome spreads" from these specimens are well established and have been extensively reviewed.[2] These will be discussed only to the extent that they are relevant to problems of cytogenetic and clinical interpretation. Although most prenatal cytogenetic studies are unambiguous, cytogenetic laboratories routinely put major efforts into discovering and interpreting the relatively rare class of "unexpected" findings including structural chromosome change and mosaicism—the presence of more than one chromosome complement (karyotype) in cell culture. In this review I will focus on the workup and interpretation of such findings. In addition, I will discuss the potential for introducing new methods into routine prenatal diagnosis and the use of cytogenetic methods in the prenatal diagnosis of several inherited genetic diseases (recessive and X-linked).

*See L. P. Shulman, MD, and S. Elias, MD, "Amniocentesis and Chorionic Villus Sampling," on pages 260-268; and N. C. Rose, MD, and M. T. Mennuti, MD, "Maternal Serum Screening for Neural Tube Defects and Fetal Chromosome Abnormalities," on pages 312-317.

Methods

Cell Culture

Culture success rates for both chorionic villus sampling (CVS) and amniocentesis are typically greater than 99%, with exceedingly low laboratory error rates (<<1%). Amniocentesis represents the first established method for fetal cell sampling and continues to provide the most common specimen type processed for prenatal chromosome analysis. Results are typically available in nine days to two weeks. Cell culture for amniotic fluid specimens is performed in either of two ways. In one, cells are randomly allocated before the production of chromosome spreads (flask technique), and in the other, cells are analyzed as components of the original colonies from which they arose (in situ technique). Each colony, in theory, will have been initiated from a single viable cell in the amniotic fluid specimen. As will be discussed, methods for interpreting mosaicism differ somewhat depending on the technique in use.[3,4] Chorionic villus sampling, being a sampling from actively proliferating tissue, affords the possibility of a "direct" chromosome preparation, with results obtained in the laboratory within two days. Because direct CVS preparations have been found to have slightly more false-negative results, particularly with respect to true fetal mosaicism, and because the chromosome structure and resolution of chromosome bands (each chromosome is identified by a highly specific longitudinal "banding" pattern) are often suboptimal, it is the standard of practice that final results are not communicated before cultured CVS specimens are analyzed.[5] The time frame for reporting results from cultured CVS specimens is similar to that for amniocentesis. At prenatal diagnostic centers with the availability of fetal blood

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ABBREVIATIONS USED IN TEXT
CVS = chorionic villus sampling
FISH = fluorescent in situ hybridization

sampling, chromosome analysis of fetal lymphocytes, by methods not fundamentally different from those used for routine postnatal chromosome analysis, allows both a rapid high-resolution chromosome study and the analysis of many independent cells derived directly from fetal tissue. This method, which entails a higher risk to the pregnancy than either amniocentesis or CVS, is used to obtain rapid (2 days) results required for pregnancy or delivery management or to address clinical ambiguities (such as mosaicism) arising from previous prenatal sampling and analysis.[6,7]

Chromosome Identification

The routine method of analysis for all specimen types is typically G banding, based on the effects of a protease (trypsin) and stain (Giemsa) on fixed chromosome preparations. In addition, cytogeneticists have at their disposal a wide array of staining techniques that allow specific questions arising during the analysis of any particular specimen to be resolved. Such questions usually arise in relation to structural chromosome abnormalities or to extreme variation in chromosomal regions known to vary somewhat in the general population. Among these methods is a powerful technique, introduced over the past several years, that allows cytogeneticists to ask specific questions about the origin of any particular chromosome region. The method—fluorescent in situ hybridization (FISH)—is based on the ability, through molecular genetic techniques, to clone—that is, to isolate and amplify—DNA specific either to a unique region of a single chromosome or to an entire chromosome (the latter used for "chromosome painting"). Such DNA is labeled with a molecule that can later be detected through the use of fluorescence microscopy. The labeled DNA, or "probe," is hybridized to a chromosome preparation from the specimen in question. Fluorescent visualization allows the cytogeneticist to determine whether the abnormal (or potentially abnormal) chromosome region is derived from the same region of chromosome (or, for painting, the same chromosome) as was the probe.[8] A limitation of this method is that it must first be determined which probes might include DNA from the region in question. Technologies now being developed but not yet available for routine use in clinical cytogenetics laboratories may overcome that limitation.[9,10]

Interphase Fluorescent In Situ Hybridization

Routine karyotype analysis is done on chromosome preparations from proliferating cells. Cells prepared for such analyses are typically blocked in the metaphase stage of mitosis with a mitotic spindle poison (colchicine) before "harvesting" for chromosome preparations. The chromosomes are present as integral structures throughout the cell cycle, but cannot be visualized in a way useful to the cytogeneticist except at metaphase. The probes used in FISH techniques are not limited to use with metaphase chromosomes. Through the use of the relevant chromosome-specific probes (for chromosomes 13, 18, 21, X, and Y), the indication for most prenatal cytogenetic analysis—increased risk of nondisjunction—can be rapidly addressed in nonproliferating interphase cells from amniotic fluid or chorionic villi.[11,12] As indicated, the standard of practice in prenatal cytogenetics is to accurately address all possible cytogenetic abnormalities in specimens, not just those related to the procedural indication. As such, the obstetrical and genetics communities generally agree that interphase FISH should not be used as a sole procedure for routine prenatal diagnostic studies, as it does not address the possible presence of unexpected chromosome abnormalities. Optimized methods for the screening of fetal cells in maternal circulation* may necessitate further exploration of that issue in the future. The issue of whether interphase FISH should be used as an adjunct to conventional cytogenetics with preliminary results (1 to 2 days) based on the method released to referring physicians is a much more controversial one.[13,14] A high success rate with the technology has been reported by one group (Integrated Genetics) on a series of 4,500 specimens.[15] The results of such studies should not be used as the sole basis for irreversible therapeutic decisions.

Problems of Interpretation

Mosaicism

The detection of mosaicism or suspected mosaicism in prenatal diagnostic specimens raises issues of two types. The first is related to the laboratory interpretation of the findings—that is, Does the finding in culture represent the true status of the amniotic fluid or chorionic villi? The second is related to the clinical importance of the findings: How likely is it that the mosaicism detected in culture is representative of the true status of the fetus? and, What is the prognosis for the fetus after the finding of true mosaicism for the abnormality in question?

Cytogenetic features. There is an extensive literature on the cytogenetic definition of mosaicism in cell culture and on methods to address suspected mosaicism.[3,4,16] The critical issue is whether the cytogenetic finding represents the status of cells present in the sampled tissue or whether it represents an artifact of cell culture (pseudomosaicism). In the flask method of amniotic fluid culture, and in chorionic villi culture, true mosaicism is defined as multiple (at least two) cells with the same abnormal karyotype present in at least two independent vessels. The in situ method for amniotic fluid has the advantage in the interpretation of mosaicism that it can be readily determined whether an abnormal cell line has arisen entirely from a single amniotic fluid colony (pseudomosaicism) or from many

*See J. Chueh, MD, and M. S. Golbus, MD, "Prenatal Diagnosis Using Fetal Cells From the Maternal Circulation," on pages 308-311.

colonies. Because the possibility always exists that a second or third abnormal colony in a single in situ preparation was actually established from a progenitor cell that floated off one original abnormal colony, there is typically an extensive search for evidence of the abnormal line in a completely independent culture (analogous to the flask method). This is particularly the case if the suspected abnormality is of known clinical significance. A large group of practicing cytogeneticists from the northeastern United States has recently published proposed guidelines for laboratories to address these issues using both the flask and in situ methods.[17]

Clinical management. Cytogenetically defined true mosaicism is detected in less than 0.5% of amniotic fluid specimens and in 1% to 2% of CVS specimens. The finding of true mosaicism in amniotic fluid culture often raises a set of difficult issues related to clinical management. Certain mosaic trisomies, in particular that for chromosome number 2, are known to be detected relatively commonly in amniotic fluid culture (and CVS) and to be without clinical consequence. Most chromosomes of the human complement have not been described in either a pure or mosaic trisomy state in the liveborn population, either from consecutive liveborn studies or from persons karyotyped because of clinical indications. The rare finding of these chromosomes as trisomies (autosomal monosomy, except for cases involving chromosomes 21 or 22, is unreported in liveborn or prenatal studies) in amniotic fluid culture will therefore typically be followed up only by noninvasive ultrasonography. Only in cases with high representation of the abnormal cell line, or when ultrasonographic findings are abnormal, is fetal blood sampling recommended as a follow-up procedure. Because discrepancies between amniotic fluid cell and fetal karyotype are rare, high levels of a clinically important abnormal cell line in mosaic state will usually be accepted as indicative of fetal status; counseling in such cases will describe the spectrum of abnormalities associated with the karyotype and inform of available reproductive options. The finding, in amniotic fluid culture, of low-level true autosomal mosaicism for a trisomy of known clinical consequence will usually serve as an indication for fetal blood sampling. Fetal blood for determining the presence of mosaicism has several advantages over amniotic fluid and CVS:

- It is, unlike the other two procedures, a direct sampling of fetal tissue;
- It allows the analysis of many (>>100, if required) metaphase cells; and
- The short-term culture assures that the cells scored are relatively independent of one another.

After normal results on fetal blood sampling, genetic counseling can be optimistic. The confirmation of low-level mosaicism in fetal blood presents a prognostic problem because there is little unbiased information available on the range of clinical effects associated with low levels of mosaicism for autosomal trisomies. Mosaic numerical abnormalities of the sex chromosomes present similar prognostic problems; unbiased prospective data are rare, and the spectrum of abnormalities associated with both the mosaic and nonmosaic states is wide, with the range of mentation (measured, for example, as IQ scores) substantially overlapping the normal range.[18] Counseling from a geneticist well versed in this area is essential for prenatally detected cases of mosaic and nonmosaic sex chromosome anomalies. Prospective studies of liveborn children with prenatally detected 45,X/46,XY mosaicism suggest that most (95%) will have normal external male genitalia but that the incidence of abnormalities of gonadal tissue structure may be increased.[19]

As noted, mosaicism is more common in CVS (both direct and cultured specimens) than in amniotic fluid cultures. This is due to a special feature of the tissue that is sampled (chorion mesenchymal core for cultured specimens and cytotrophoblast for direct) and has been termed confined placental mosaicism.[20] This phenomenon represents mosaicism limited to the extraembryonic membranes and is not detected in fetal tissues; it occurs in 1% to 2% of CVS cultures.[5,21,22] A growing set of data suggests that confined placental mosaicism, while not associated with fetal karyotypic abnormality, may be associated with intrauterine growth retardation and perinatal loss.[23,24] Although information is continuing to be collected in this area, amniocentesis is often recommended as a follow-up procedure after the discovery of CVS mosaicism. If the mosaicism involves a clinically important trisomy, fetal blood sampling may also be recommended. Strong evidence exists, indeed, that placental mosaicism involving a normal cell line along with a cell line trisomic for chromosome 13 or 18 may be an important etiologic factor in the survival to term of some fetuses fully trisomic for chromosome 13 or 18.[25] The normal cell line apparently arises by the loss of a chromosome from an originally trisomic cell line. In about a third of such cases both homologues of either chromosome 13 or 18 will have arisen from one parent, the contribution from the second parent having been lost. This is known as uniparental disomy and has come to be recognized as a factor in human morbidity over the past several years.[26] In at least one reported case, confined placental mosaicism for trisomy 15 (not a viable fetal trisomy) has been followed by the birth of a child affected with the Prader-Willi syndrome—a syndrome known to be associated with maternal uniparental disomy (no paternal contribution) of chromosome 15.[27] In addition, uniparental disomy for chromosome 16 has been presented as a possible cause of the morbidity associated with confined placental mosaicism of chromosome 16.[24] This raises an as-yet-unresolved diagnostic question: Should molecular evidence for uniparental disomy be pursued in cases of confined placental mosaicism involving chromosomes known to cause morbidity when present in the uniparental disomic state?

Prognostic Issues in Cytogenetics

The woman or couple referred for a prenatal diagnosis because of an increased risk of autosomal trisomy can

expect the result to be relatively "straightforward," either normal or trisomic, trisomy for chromosome 21 being, by far, the most commonly detected abnormality. Several areas in which counseling is more complex have been discussed earlier, including mosaicism and sex chromosome abnormality. Several other findings, all of which fall into the category of "unexpected chromosome abnormalities" are discussed further. These include both extra, de novo (noninherited) "marker" chromosomes and de novo apparently balanced structural rearrangements. In both cases, as with mosaic trisomy, the counselor must present a risk associated with the abnormality. These tend to be difficult counseling issues for patients who were expecting either clearly normal or abnormal results.

Marker chromosomes. Marker chromosomes is a term used to describe small accessory chromosomes, typically of unknown origin. Such chromosomes are often present in a mosaic state. A proportion of marker chromosomes are found to be inherited from normal parents and impart no increased risk to the pregnancies. In about 1 of 2,500 prenatal diagnoses, a de novo marker chromosome is discovered.[28] In a proportion of such cases, particularly with the increased availability of molecular probes, the chromosome can be defined as one known to be consistently associated with a clinical syndrome.[29,30] For markers that appear to have a substantial amount of chromosome material staining as "euchromatin" (gene-encoding regions), the prognosis is typically poor.[31] For most markers, however, geneticists must rely on empirical risk figures derived from the limited number of cases in which markers were ascertained prenatally, the pregnancy maintained, and clinical follow-up achieved.[28] The risk for some abnormality in a newborn tends to fall between 10% and 15%, but has wide confidence limits. The ability to define more precisely the chromosomal makeup of marker chromosomes may ease this difficult counseling issue in the future.[32]

De novo apparently balanced rearrangements. Balanced chromosome rearrangements are a class of structural chromosome change in which all of the genetic material is present, but its location or orientation has changed. Cytogenetics laboratories commonly identify carriers of balanced chromosome rearrangements, most often translocations, due to either multiple pregnancy loss, infertility, or by chance during prenatal diagnostic studies. In the last case, a balanced rearrangement discovered in a fetus is found to be inherited from one of the parents. Such a rearrangement does not entail an increased risk for physical or mental abnormality to the fetus. The carrier of such a rearrangement is, however, at an increased risk for a chromosomally unbalanced fetal karyotype detected at prenatal diagnostic studies or at birth. The actual level of risk depends on the method of ascertaining the rearrangement (the previous birth of a liveborn chromosomally unbalanced child carries the highest risk) and on the precise nature of the chromosome change (the smallest amount of imbalance carries the highest risk). Several schemata for calculating such risks have been developed.[33,34]

A more difficult counseling issue arises when an apparently balanced rearrangement is discovered de novo at the prenatal diagnosis. Within institutions for the mentally handicapped, when persons with known clinical diagnoses are eliminated from consideration, about a tenfold higher incidence is found of apparently balanced rearrangements than in the general population.[35] This information, however, simply indicates that there is an increased risk to the fetus discovered to carry such a translocation. The biased data set cannot provide an actual risk figure. As was the case for marker chromosomes, only prospective data gathered at prenatal diagnostic studies with proper follow-up are suitable for calculating the risk. Data of Warburton collected from cytogenetics laboratories throughout the United States and Canada suggest a risk for a fetal abnormality of between 3% and 10% (to be compared with the usual estimate of 2% to 3% for congenital malformations at birth in the general population).[28] The mechanism(s) responsible for the increased risk associated with apparently balanced rearrangements is unknown. The abnormal clinical features, often mental retardation without major dysmorphic findings, are not those that would be expected if the chromosome breaks associated with the translocations were causing new dominant mutations. It may be that the rearrangements are not truly balanced, but have caused a loss or gain of genetic material not visible at the cytogenetic (light-microscopic) level or that the actual position of the genetic material, and not solely its content, is critical.

Analysis of Genetic Diseases With Cytogenetic Manifestations

To this point, discussion has focused on indications for, and interpretations of, cytogenetic studies related to the determination of a fetal karyotype. Such studies represent the great majority of all prenatal cytogenetic analyses. For several inherited genetic diseases, however, though the risk for a karyotypic abnormality is not increased, cytogenetic methods are used in prenatal (and postnatal) diagnostic studies. Cells of affected persons show either generalized or site-specific effects on chromosomes through which the diseases may be diagnosed.

Chromosome Breakage Disorders

A series of three relatively rare autosomal recessive diseases are usually classified together as the "chromosome breakage disorders." They are the Bloom syndrome, Fanconi's anemia, and ataxia telangiectasia. Although the actual clinical presentations of the diseases differ markedly one from the other, all share a tendency toward spontaneous chromosome breakage (with different patterns in each disease) and a predisposition toward the early development of cancers (of different types in each disease).[36] Analysis for the Bloom syndrome is aided by a tendency of chromosomes from patients to show greatly increased numbers of "sister chromatid exchanges."[37] Special culture conditions and staining techniques are required to visualize sister chromatid exchanges. Prenatal diagnostic studies for ataxia telangi-

ectasia take advantage of a hypersensitivity to x-ray induced damage, whereas those for Fanconi's anemia rely on hypersensitivity to a class of chemical mutagen known as alkylating agents.[38,39] Because the successful treatment of persons affected by Fanconi's anemia often depends on the presence of an HLA-identical hematopoietic stem cell donor, families with an affected child will usually have the option to have HLA typing as well as prenatal diagnostic studies in future pregnancies. Cord blood collected from HLA-identical unaffected siblings provides an excellent source of donor cells.[40]

Roberts's Syndrome

Another recessively inherited disorder, Roberts-SC phocomelia syndrome (or pseudo-thalidomide syndrome), usually lethal in the prenatal or neonatal period, is characterized by severe limb reduction abnormalities and, when measurable, profound mental retardation. (There are reports of less severely affected persons; it is unknown if these represent mutations at the same genetic locus as those with the more severe manifestations.) By cytogenetic analysis, Roberts's syndrome is recognized by an apparent repulsion of sister chromatids at blocks of heterochromatin (noncoding regions), usually near the centromeres. This, combined with ultrasonographic findings, serves as an effective means for prenatal diagnosis of the disorder.[41]

Fragile X Syndrome

The fragile X-linked mental retardation syndrome represents the single most common genetically inherited mental retardation syndrome and, after the Down syndrome, the single greatest contributor to mental retardation in the population. Readers are referred to an excellent recent review of its clinical, genetic, and molecular details.[42] The disease received its name from its association with an inducible "fragile site" near the end of the long arm of the X chromosome. The site is induced through the manipulation of cell culture conditions and is expressed in a variable percentage of cells from most affected and some carrier persons. With the cloning of the gene for this disorder (*FMR1*, for fragile X-linked mental retardation-1), that approach has been largely superseded by molecular technologies involving the direct analysis of the gene. Such technologies have greatly aided carrier testing and prenatal diagnostic studies. Mention of the disease is included here both for historical reasons and because there remains some use of the cytogenetic methods in the prenatal assignment of risk for mental retardation for female carriers of the "full mutation" for fragile X (the mutation exists in "premutation" and "full mutation" forms).[43]

Conclusions

Cytogenetic analysis due to an increased risk for nondisjunction is the most commonly used method requiring invasive procedures for prenatal diagnosis. Highly accurate and successful systems for such analyses are established, and developments in molecular genetics have become well integrated into those systems. That integration continues to shed new light on old problems and to allow the introduction of entirely new approaches to prenatal diagnosis. The analysis of fetal cells in maternal circulation holds promise of fundamentally changing access to these services and of perhaps necessitating a serious look at the question of whether full karyotypic analysis should remain the standard of practice over the long term. These developments promise an exciting future in both the diagnostic-research and the medical-societal arenas.

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